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ISOLATION, IDENTIFICATION AND QUANTIFICATION OF BIOACTIVE COMPOUNDS FROM *AMARANTHUS HYPOCHONDRIACUS* LEAF EXTRACT: A VITAL SOURCE OF POTENT NATURAL ANTIOXIDANTS

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Keywords:

A. hypochondriacus leaves, GC-MS, metabolite profiling, Alkylated phenols, HPLC, NMR, DPPH assay

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ABSTRACT: In this study we aimed at isolation, identification, and quantification of two phenolic compounds reported for the first time in leaves of *Amaranthus hypochondriacus.* A non targeted GC-MS metabolite profiling of hexane extracts of amaranth leaves was done which indicated the presence of major phenolic compounds. Two alkylated phenols namely 2,4-di-*tert*-butyl phenol (A) and 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl) propanoic acid (B) were isolated for the first time from the hexane extract of *A. hypochondriacus* leaves. Structure elucidation of both the compounds was done using ^{1}H NMR and purity was checked by GC-MS. Quantitative estimation of purified compounds in leaves extract was carried out using RP-analytical HPLC. The antioxidant potential of the isolated compounds (A), (B) and the hexane extract of leaves were investigated using *in-vitro* 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radicals scavenging bioassay. Hexane leaf extract (IC_{50} = 385.34 ± 37.11 µg/mL), 2,4-di-*tert*-butyl phenol (A) (IC_{50} = 253.76 ± 24.67 µg ml^{-1}) and 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl) propanoic acid (B) (IC_{50} = 174.09 ± 16.89 µg ml^{-1}) exhibited significant free radicals scavenging activity. Thus, our study reports high content of two alkylated phenols for the first time in *Amaranthus hypochondriacus* leaves extract. It substantiates the usage of amaranthus leaves as a prominent source of antioxidant bioactive potent enough to substitute synthetic toxic antioxidants in food and pharmaceutical industries.

INTRODUCTION: An increase in the incidence of degenerative diseases such as diabetes, Alzheimer's, cancer, *etc*. has diverted the attention of the researchers towards the urgent need for reducing oxidative stress occurring in body cells using antioxidants [1].

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Owing to this there has been a huge surge in the demand for naturally occurring antioxidants which are effective in quenching free radicals and in inhibiting oxidative chain reactions. Amaranthus is one such plant that is a vital source of dietary antioxidant phytonutrients [2].

It is an ancient plant belonging to the family of Amaranthaceae which finds its origin from Central and South America where it was grown for its seeds by the ancient Aztecs, Incas, and Mayas. Amaranthus is much valued for its nutritive potential as they are rich in proteins and free essential amino acids such as lysine, starch,

minerals and lipids, vitamins and dietary fiber [3, 4]. The plant is also rich in secondary metabolites such as phenolics [5]. Phenolic compounds are secondary metabolites arising biogenetically from the shikimate/phenylpropanoid pathways producing monomeric and polymeric phenols and polyphenols. These compounds consist of many classes like flavonoids, tannins, chalcones, coumarins, *etc*. in which phenolic acids form one-third of the phenolic compounds in the human's diet [6]. Phenolic compounds have been reported as potential anti-allergenic, anti-atherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic properties [7, 8, 9].

Metabolomics is an important tool that aids in knowing about the diverse metabolites present in plants [10]. GC-MS, LC-MS, and NMR spectroscopy have been successfully used in plant metabolomics for the identification of unknown and unknown metabolites [11]. Literature provides very less information about the lipophilic phenolic compounds present in non-aqueous extracts of amaranthus. Hence in this study, we aimed at isolating and identifying bioactive compounds and fractions from hexane extracts of *Amaranthus hypochondriacus* leaves using sophisticated analytical techniques like GC-MS, HPLC, NMR, and mass spectrometry. To, the best of our knowledge, we report for the first time two alkylated phenols namely 2,4-di-tert-butyl phenol and 3- (3, 5- di- tert- butyl- 4- hydroxyphenyl) propanoic acid in *A. hypochondriacus*. Estimation of their free radical scavenging potential was done in order to assess the antioxidant potential of isolated and purified alkylated phenolic compounds and the hexane extract of the leaves. Hence, through our study, we establish the Amaranth leaves as a strong natural antioxidant candidate.

MATERIALS AND METHODS:

Chemicals and Reagents: All the solvents used for extraction and HPLC analysis were of Qualigens make. Silica Gel Preparative TLC plates (20 × 20 cm), were from s.d. fine-chem Ltd., Mumbai. All other chemical standards and reagents *e.g.* squalene, methoxyaminehydrochloride, pyridine and n-methyl-n-trimethylsilyltrifluoro-acetamide (MSTFA) for GC-MS analysis, Butylated hydroxytoluene (BHT) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) for antioxidant assay were purchased from Sigma Chemical Company, USA.

Plant Material: Ten *A. hypochondriacus* leaves accessions were collected from experimental research field of the Integral University, Lucknow. The passport data of all the collected accessions of the plant sample was identified and validated at the Pharmacognosy Department of Faculty of Pharmacy, Integral University. The plant specimens were authenticated and kept as voucher specimen in the Herbarium. The leaf samples were lyophilized until a constant mass was obtained.

The lyophilized samples (1 g each) of all the ten accessions were extracted with 250 mL hexane. The extraction process was repeated twice with 250 mL solvent and the combined extracts were filtered and the filtrate was concentrated under reduced pressure using rotavapour. The residues so obtained were air-dried and stored at -20 °C until further analysis.

Isolation and Purification of Two New Compounds: The hexane extract of amaranth leaves was dissolved in ethanol (500 mg/mL) and spot application was done on a preparative TLC plate (20 × 20 cm) coated with silica gel Si 60 F_{254}. The chromatographic plate was developed in a solvent system Hexane-Ethyl acetate (95:5) giving prominent band separation as perceived under UV radiation. Both the compounds A and B were clearly separated in two distinct bands which were isolated from the chromatographic plate. The purified compounds A and B were used for further analysis of structure elucidation done by NMR and mass spectrometry, purity checked by GC-MS and quantified by HPLC [12, 13].

GC-MS Analysis: GC-MS analysis was conducted on the amaranth leaf extracts and the purified compounds A and B by preparing a volatile trimethylsilyl derivative of the samples using MSTFA as a derivatized by the method as reported in literature [14]. Xcalibur software was used to process the chromatographic and mass spectrometric data so obtained. The concentration of metabolites was calculated on the percent peak area basis.

HPLC Analysis: HPLC–PDA analysis was carried out in which both the purified compounds (A) and

(B) were detected at 278 nm using an isocratic mobile phase consisting of acetonitrile-water (70:30) at a flow rate of 1.0 mL min^{-1} with a C_{18} (3.9 × 300 mm) reverse-phase analytical column. The peaks were confirmed and quantified by using purified fractions of 2,4-di-tert-butyl phenol and 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propanoic acid [15, 12].

NMR Analyses: ^{1}H NMR and 2D NMR of purified compounds were recorded on 300 MHz NMR spectrometer by dissolving compounds in 500 µL $CDCl_3$ in 5 mm NMR tubes. To confirm the assignments, two-dimensional Correlation spectroscopy COSY), Heteronuclear single quantum coherence (HSQC) and Heteronuclear multiple bond correlation (HMBC) experiments were performed using Bruker's standard pulse program library.

Determination of Antioxidant Activity *via* 1,1-diphenyl-2-picrylhydrazyl (DPPH) Based Free Radical Scavenging Activity: The leaf extract with the highest phenolic content and compounds A and B were dissolved in methanol and sample solutions of different concentrations were made for the determination of antioxidant activity. For assay, 50 µL of sample solution was added to 5 mL of 0.004% methanol solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) and incubated at 37 °C for 30 min. The absorbance was measured at 517 nm. Inhibition percent of DPPH was determined by earlier reported method [16, 17]. Half maximal inhibitory concentration (IC_{50}) values represent the concentration of compounds to scavenge 50% of DPPH. The assay was carried out in triplicate. BHT was used as positive control [18].

RESULTS AND DISCUSSION:

Identification and Purity Determination of Isolated Phenolic Compounds by GC-MS: GC-MS based metabolite profiling of amaranth leaves hexane extract was done as shown in **Fig. 1**. The analysis showed the presence of 28 non aqueous metabolites **Table 1,** out of which compounds A and B showed highest content. Compound A showed elution at retention time (RT) 19.59 min and compound B at 32.24 min. The relative concentration of compounds A and B was found to be 17.40% and 13.41% respectively. The in-built mass library suggested the two major metabolites, compounds A and B as unknown phenolics and hence were targeted for isolation, identification, and quantification.

The GC-MS analysis was utilized to estimate the purity of the isolated compounds A and B **Fig. 2** and found to be 100% pure. The mass spectra of compound A exhibited a base peak [M + H]− at m/z 207 and a base peak [M+H]+ at m/z 279 for compound B. The GC-MS library suggested the two compounds to be alkylated phenols. The structural identification of these compounds was further confirmed by NMR.

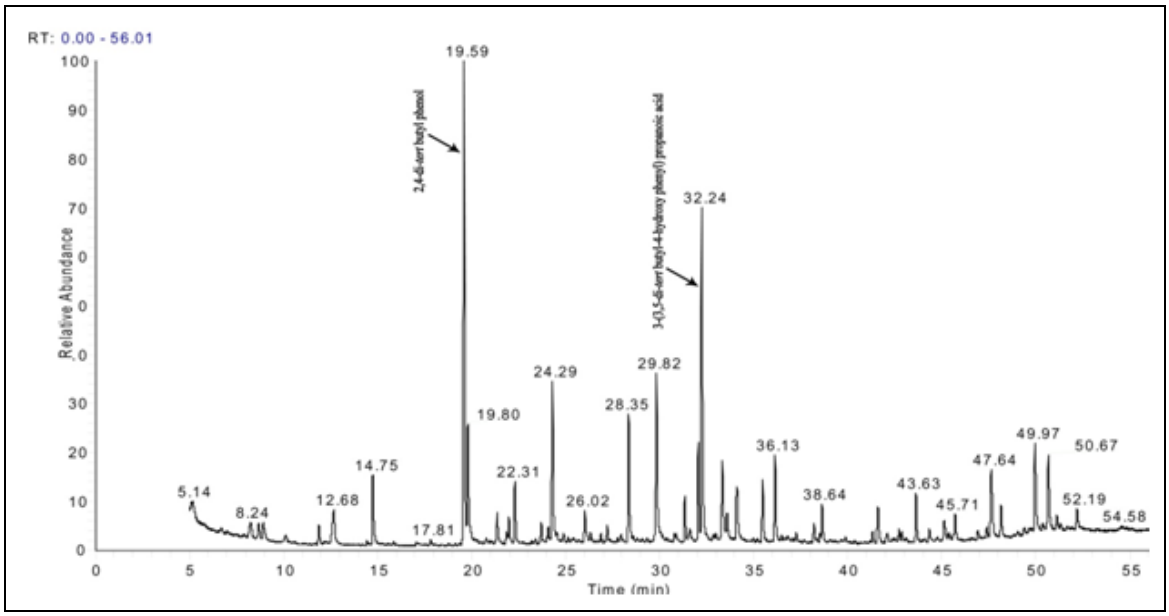


FIG. 1: GC-MS CHROMATOGRAM OF NON-AQUEOUS METABOLITES IN EXTRACT OF LEAVES OF *A. HYPOCHONDRIACUS*

TABLE 1: QUALITATIVE AND QUANTITATIVE ESTIMATION OF NON-AQUEOUS METABOLITES IN EXTRACT OF LEAVES OF *A. HYPOCHONDRIACUS* USING GC-MS CHROMATOGRAPHY

Metabolites	Mass Fragmentation	tR (min)	Relative conc. (%)
Butanoic acid	m/z 145(M+), 130(M+-CH_3), 117, 73, 61, 43	8.24	1.35±0.01
Glycerol	m/z 308(M+), 293(M+-CH_3), 205, 147, 133, 103, 101, 73	10.13	0.59±0.03
Phosphoric acid	m/z 329(M+), 314(M+-CH_3), 299, 211, 177, 159, 101, 89.73	12.66	3.2±0.29
Tetradecene	m/z 308(M+), 293(M+-CH_3), 205, 147, 133, 103, 101, 73	14.73	3.37±0.35
2,4-ditert-butyl phenol	m/z 278 (M+), 268(M+-CH_3), 263, 207, 91, 73	19.59	17.40±1.31
Cetene	m/z 226(M+), 183(M+-CH_3), 127, 113, 85, 71, 57, 43	19.8	3.51±0.31
Nonadecene	m/z 266(M+), 236(M+-CH_3), 167, 139, 125, 111, 97, 57, 43	24.3	7.18±0.66
Myristic acid	m/z 278 (M+), 268(M+-CH_3), 263, 207, 91, 73	26.01	1.30±0.11
Eicosene	m/z 280(M+), 252(M+-CH_3)+E12, 182, 111, 97, 83, 69, 57, 43	28.34	5.51±0.43
Palmitic acid	m/z 328(M+), 313(M+-CH_3), 285, 269, 243, 117, 73	29.82	7.73±0.65
trans-farnesol	m/z 294(M+), 279(M+-CH_3), 257, 229, 143, 107, 73, 69.	31.34	1.76±0.13
Docosene	m/z 308 (M+), 293 (M+-CH_3), 280, 139, 125, 111, 69, 57	32.06	3.83±0.35
3,5-ditert butyl-4-hydroxy propanoic acid	m/z 294(M+), 279(M+-CH_3), 135, 105, 73	32.24	13.41±1.18
Stearic acid	m/z 308(M+), 293(M+-CH_3), 205, 147, 133, 103, 101, 73	33.34	4.00±0.36
Linoleic acid	m/z 352(M+), 337(M+-CH_3), 262, 129, 95, 73	33.59	0.90±0.06
Linolenic acid	m/z 350(M+), 335(M+-CH_3), 280, 149, 129, 95, 73	34.11	3.41±0.28
Hexacosene	m/z 364(M+), 181(M+-CH_3), 153, 125, 97, 83, 71, 57, 43	35.46	2.66±0.23
Monopalmitin	m/z 474(M+), 459(M+-CH3), 371, 239, 205, 147, 73	38.53	0.20±0.01
Pentatriacontene	m/z 492(M+), 407(M+-3), 337, 253, 183, 141, 113, 99, 85, 71, 57, 43	38.63	1.33±0.11
Hentriacontane	m/z 506(M+), 448(M+-H3), 365, 309, 253, 197, 155, 113, 99, 71, 57	42.73	0.53±0.04
Squalene	m/z 410, 395(M+- CH_3), 341, 203, 191, 136, 81, 69	43.63	2.14±0.18
Hexacosanoic acid	m/z 506(M+), 448(M+-H3), 365, 309, 253, 197, 155, 113, 99, 71, 57	45.13	1.25±0.11
Octacosanol	m/z 506(M+), 448(M+-H3), 365, 309, 253, 197, 155, 113, 99, 71, 57	45.71	1.04±0.09
Cholesterol	m/z 458(M+), 443(M+-CH_3), 368, 329, 247, 129, 73	47.38	0.38±0.02
α-Tocopherol	m/z 502(M+), 397(M+-CH_3), 277, 237, 73	47.64	4.36±0.34
Triacontanol	m/z 506(M+), 448(M+CH_3), 365, 309, 253, 197, 155, 113, 99, 71, 57	48.15	1.53±0.12
Stigmasterol	m/z 484(M+), 394(M+-CH_3), 255, 217, 147, 129, 73	49.95	4.72±0.42
α-Amyrin	m/z 498(M+), 483(M+-CH_3), 393, 279, 218, 189, 122, 73	51.11	0.68±0.05

Mean values ± SD of the relative concentration of metabolites

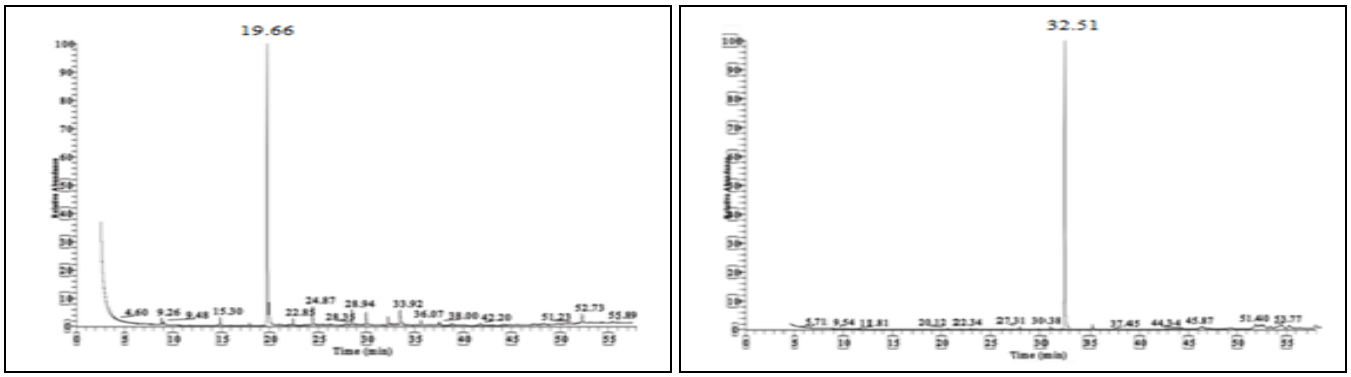
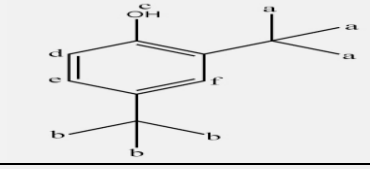
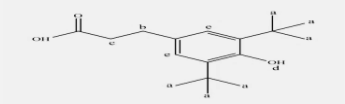


FIG. 2: GC-MS CHROMATOGRAM OF PURIFIED 2,4-DI-TERT-BUTYL PHENOL AND 3-(3,5-DI-TERT-BUTYL-4-HYDROXYPHENYL) PROPANOIC ACID RESPECTIVELY

Structure Elucidation of Compounds A and B *via* NMR Spectroscopy:

TABLE 2: 1D AND 2D NMR ASSIGNMENTS OF COMPOUND A and B

2,4- di-*tert*-butyl Phenol	Position	Proton (δ_H in ppm)	
	a	1.29 (9H, s)	
	b	1.41 (9H, s)	
	c	4.68 (1H, s)	
	d	6.60 (1H, d, *J* = 7.95 Hz)	
	e	7.07 (1H, dd, *J* = 5.96, 2.26 Hz)	
	f	7.29 (1H, d, *J* = 2.33 Hz)	
3-(3,5-di-*tert*-butyl-4-hydroxyphenyl) propanoic acid	**Position**	**Proton (δ_H in ppm)**	**HSQC(δ_C in ppm)**
	a	1.42 (18H, s)	C-1 (29.92)
	b	2.64 (2H, t)	C-2 (30.20)
	c	2.87 (2H, t)	C-3 (30.57)
	d	5.08 (1H, s)	-
	e	7.00 (2H, s)	C-4 (124.8)

Compound A: It was isolated as yellow solid and elucidated as 2,4-di-tert-butyl Phenol with the molecular formula of $C_{14}H_{22}O$ by ESI-MS (+ve mode) showing molecular ion peak [M+H]+ at m/z 207 (calculated for $C_{14}H_{22}O$, 206.17) **Fig. 3A**.

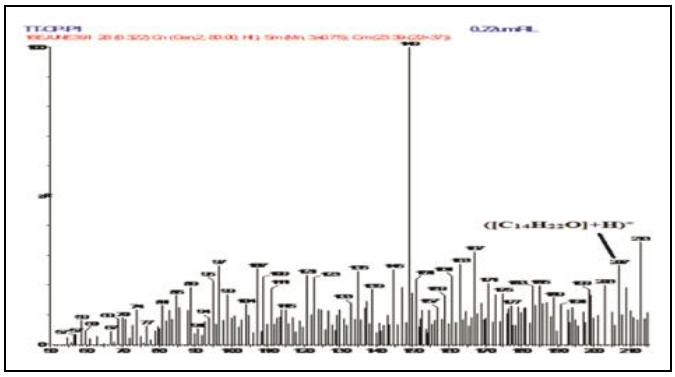


FIG. 3A: ESI-MS SPECTRUM OF 2,4-DI-TERT-BUTYL PHENOL

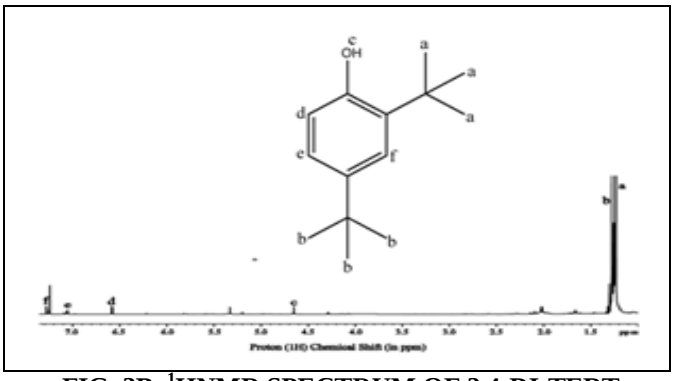


FIG. 3B: ^{1}HNMR SPECTRUM OF 2,4-DI-TERT-BUTYL PHENOL

Its ^{1}H NMR spectrum **Table 2** showed three signals in aromatic region, a doublet (J = 2.33 Hz) at 7.29 ppm (H-f) due to one meta-aromatic proton fused between di-tert-butyl groups, a double doublet (J = 5.96, 2.26) at 7.07 ppm (H-e) due to one meta-aromatic proton and a doublet (J = 7.95Hz) at 6.60 ppm (H-d) due to one ortho-aromatic proton of benzene ring. The singlet at 4.68 ppm (H-c) showed the presence of one hydroxyl group. Two singlets of di-tert-butyl groups were at 1.41 ppm (9H-b) and 1.29 ppm (9H-a) **Fig. 3B**. The NMR assignments of 2,4-di-tert-butyl-phenol were compared with those in the earlier reports [15, 13].

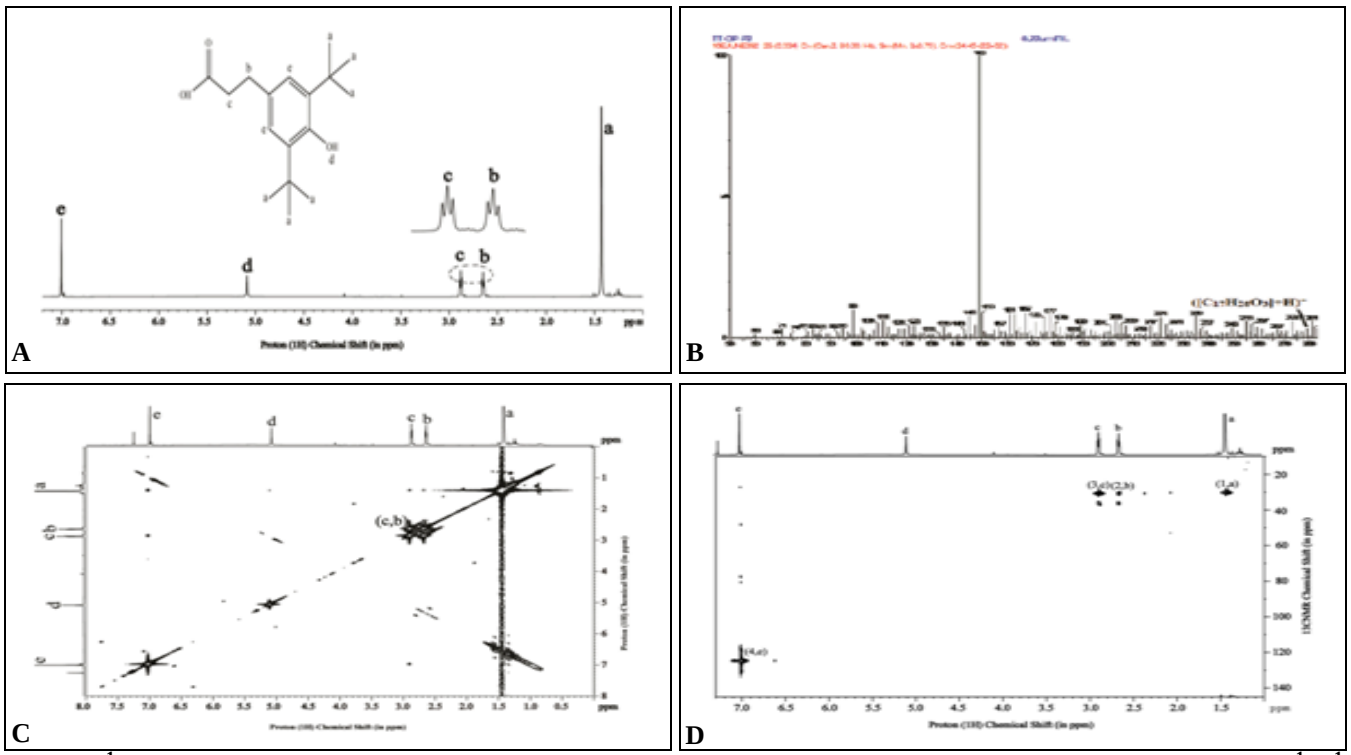


FIG. 4A: ^{1}H NMR SPECTRA OF COMPOUND B, 4B: ESI-MS MASS SPECTRA OF COMPOUND B, 5C: ^{1}H-^{1}H COSY NMR SPECTRA OF COMPOUND B, 5D: HSQC NMR SPECTRA OF COMPOUND B

Compound B: It was isolated as white crystals and elucidated as 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propanoic acid with the molecular formula of $C_{17}H_{26}O_3$ by ESI-MS (+ve mode) showing molecular ion peak [M+H]+ at m/z 279 (calcd. for $C_{17}H_{26}O_3$, 278.19) **Fig. 4B**. It showed to be the substituted derivative of compound A as it displayed somewhat similarity in NMR spectrum.

The ^{1}H NMR spectrum **Table 2** showed only one signal in the aromatic region as other aromatic carbons were substituted with some groups. It showed singlet at δH 7.00 (H-e) due to two ortho aromatic protons. The singlet at δH 5.08 (H-d) was because of presence of one hydroxyl group which was confirmed by shaking the pure fraction-4 with D_2O. Two methylene groups showed triplets at δH 2.87 (2H-c) and δH 2.64 (2H-b). In this all the di-tert-butyl protons are identical so a singlet of 18 protons was observed at δH 1.42 (H-a) **Fig. 4A**. A correlation was observed in the ^{1}H-^{1}H COSY spectrum between methylene protons H-c (δH 2.87) /H-b (δH 2.64) **Fig. 4C**. The ^{13}C-^{1}H correlations of fraction-4 were confirmed by HSQC. The aromatic hydrogen at δH 7.00 (H-e) showed correlation with aromatic carbon at δC 124.8 (C-4), two methylene group hydrogen at δH 2.87 (2H-c) and δH 2.64 (2H-b) with two methylene carbons at δC 30.57 (C-3)and δC 30.30 (C-2) and all the identical di-tert-butyl protons at δH 1.42 (H-a) with alkyl carbon at δC 29.92 (C-1) **Fig. 4D**. A synthetic derivative of 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propanoic acid has been reported to be highly susceptible to oxidation [19], however, its natural occurrence has not been reported so far.

HPLC Quantification: Quantitative estimation of purified alkylated phenolic compounds of hexane extracts of amaranth leaves was carried out using RP-analytical HPLC. The highest concentration of 2,4-di-tert-butyl phenol and of 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propanoic acid was quantified as 21.97 ± 2.14 mg/g dry wt and 16.48 ± 1.56 mg/g dry wt. of leaves of *A. hypochondriacus* respectively.

Antioxidant Potential: Antioxidant activity of Amaranthus leaf extract and isolated compounds is due to antiradical activity which is assayed in the present study as discoloration and measurement of decreasing quantity of DPPH free radicals. The purified compounds 2,4-di-tert-butyl phenol and 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propanoic acid and the crude hexane extract of the Amaranthus leaves were evaluated for antioxidant activity with (BHT) as external standard **Fig. 5**. Two alkylated phenols showed promising DPPH free radical scavenging potential. The 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propanoic acid was found to be more effective (IC_{50} = 174.09 ± 16.89 µg/ml) as compared to that of 2,4-di-tert-butyl phenol (IC_{50} = 253.76 ± 24.67 µg/ml). The crude leaf extract (IC_{50} = 385.34 ± 37.11 µg/ml) exhibited appreciable antioxidant capacity [20].

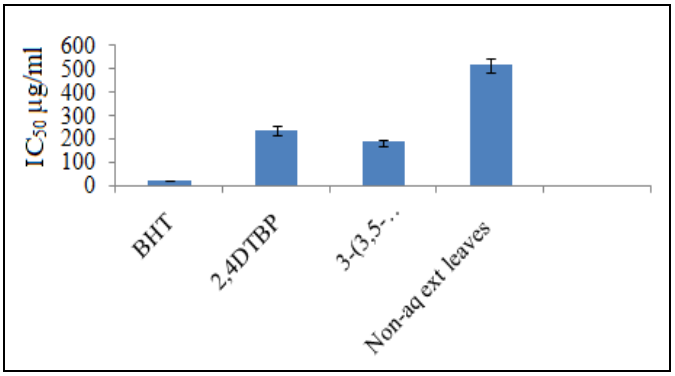


FIG. 5: DPPH BASED FREE RADICAL SCAVENGING ACTIVITY OF STANDARDS, COMPOUND A, B, AND CRUDE HEXANE EXTRACT OF LEAVES OF *A. HYPOCHONDRIACUS*

CONCLUSION: To the best of our knowledge, we report for the first time two new alkylated phenols, 2,4-di-tert-butyl phenol and 3-(3,5-di-tert-butyl-4-hydroxyphenyl) propanoic acid which was isolated and identified from the hexane extract of amaranthus leaves. The NMR, GC-MS and HPLC techniques were employed to confirm their presence. Purified phenols and leaf extracts were found to be effective in scavenging DPPH radical *in-vitro*. A high amount of these alkylated phenols serve as new alternative for many health/diet recommendations and as a potential ingredient for nutraceutical and pharmaceutical products. This is an important finding which will further increase the marketability and importance of this plant as a lucrative source of antioxidant bioactive in food, cosmetics, pharmaceutical, and nutraceutical industry.

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CONFLICTS OF INTEREST: Authors declare no conflicts of interest.

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